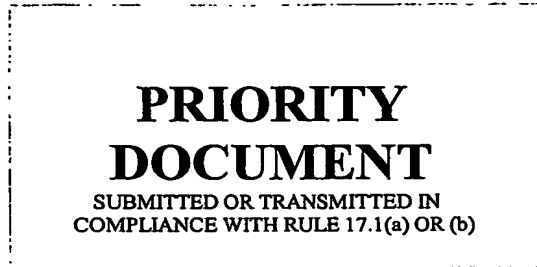
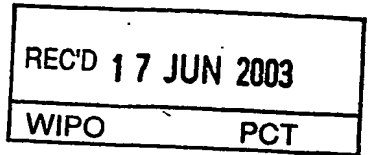


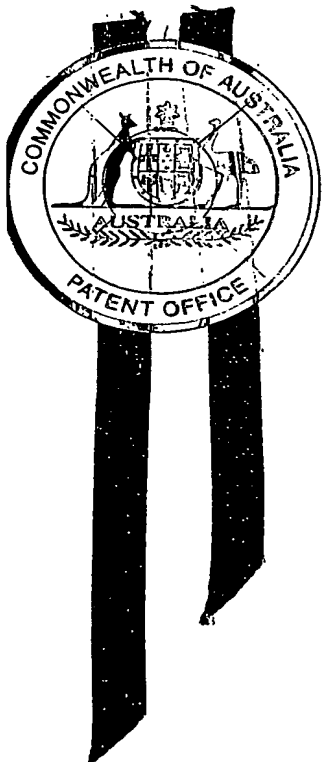


Rec'd PCT/PTO 29 NOV 2004  
PC/AU03/00660



Patent Office  
Canberra

I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951222 for a patent by APOLLO LIFE SCIENCES PTY LIMITED as filed on 05 September 2002.



WITNESS my hand this  
Tenth day of June 2003

*J. Billingsley*

JULIE BILLINGSLEY  
TEAM LEADER EXAMINATION  
SUPPORT AND SALES

BEST AVAILABLE COPY

Our Ref: 7728560

P/00/009  
Regulation 3:2

AUSTRALIA

Patents Act 1990

**PROVISIONAL SPECIFICATION**

**Applicant(s):** Apollo Life Sciences Pty Limited  
34 Cook Street  
Randwick New South Wales 2031  
Australia

**Address for Service:** DAVIES COLLISON CAVE  
Patent & Trade Mark Attorneys  
Level 10, 10 Barrack Street  
SYDNEY NSW 2000

**Invention Title:** Cell fusion

The invention is described in the following statement:

## **CELL FUSION**

### **Background of the Invention**

The present invention relates to a method and apparatus for fusing first and second cells,  
5 and in particular, for producing hybrid cells by electrofusion.

### **Description of the Prior Art**

The reference to any prior art in this specification is not, and should not be taken as, an  
acknowledgement or any form of suggestion that the prior art forms part of the common  
10 general knowledge in Australia.

Previously it has been known to fuse cells together using a variety of techniques such as  
chemical fusion employing polyethylene glycol, biological methods such as viruses or  
viral proteins or electrofusion of cells in suspension. Methods of fusion carried out by  
15 chemical or biological means often suffer from problems associated with contamination,  
low efficiency and cytotoxicity.

There are a number of advantages to using electrofusion for producing hybrid cells. The  
fusion conditions can be better controlled and optimised depending on the type of cell to be  
20 fused than chemical or biological fusion allows. This allows electrofusion to lead to an  
increase in cell fusion efficiency.

The basis for electrofusion is to expose pairs of cells, in close membrane contact, to an  
electric field that induces a sufficient voltage across their cellular membrane to cause  
25 mechanical breakdown of the cell membrane and the formation of pores at the point of  
cell-to-cell contact. Ideally the pores should be of sufficient size to allow transfer of  
cellular material between the two cells, particularly allowing the two nuclei to come  
together and subsequently fuse. This formation of pores should also be reversible such that  
any pores formed at points other than that of cell-to-cell contact seal quickly.

30

A further process known as dielectrophoresis (DEP) in which a non-uniform alternating

- 2 -

electric field is applied to the cells in order to ensure good cellular contact prior to fusion. Typically DEP is employed to form 'pearl chains' of large numbers of cells between the electrodes in the fusion container.

5 Whilst electrofusion has a number of advantages over chemical and biological processes, all of these methods fuse cells simultaneously in large numbers. The pairing of the cells, necessary to form hybrid cells, is therefore completely random. Accordingly, the current methods are limited in that they require a chemically sensitive immortal cell such that unfused and self-fused cells can be eliminated from the final culture.

10

Furthermore, these 'bulk' methods also do not lend themselves to hybrid creation from rare cells. Typically fusion methods require millions of cells in order to overcome the problems associated with ensuring cellular contact between the desired cells. In some cases the number of target cells for fusion might only number in 10's-100's. Further, in 15 the recovery phase, whereby cells from the fusion process are plated out and the chemical selection process to remove unfused cells takes place, there is no guarantee of clonal purity in the final product. This plating of cells is also extremely time consuming.

It would therefore be desirable to provide a method whereby lesser numbers of cells could 20 be selected, electrically fused and recovered to grow.

An example of a system suitable for performing electrofusion on a small number of cells is described in WO93/05166. This describes apparatus that utilises an electrode coated with ligands. In use, the ligands are used to attract target cells bearing complimentary ligands. 25 Once the ligands are bound, the cells are therefore effectively bound to the electrode. Accordingly, at this point the target cells can be brought into contact with partner cells allowing the cells to be fused.

However a number of drawbacks exist with these techniques. Firstly, the target cell is held 30 in contact with the electrode during the electrofusion process. As a result the cell is usually subject to an intense electric field which tends to damage the cell. Secondly, the

- 3 -

technique can only be performed with a number of target cells attached to the electrodes, and a number of partner cells. Accordingly, this means that any cells successfully fused may be separated out from cells that do not fuse, which can be a complex procedure. A further disadvantage of this technique is that cells can bind to the electrode non-specifically leading to false fusion events taking place.

A second example of a system for performing cell fusion on individual cells is described in WO01/09297. In this example, cells are manipulated using a combination of optical trapping, and pushing the cells with micro-electrodes. Once the cells are correctly positioned relative to each other, an electric field is applied to the cells to cause the cells to fuse.

However, a number of significant drawbacks exist with the apparatus. Firstly, the presence of the laser and associated optics required to manipulate the cells results in the apparatus being expensive, time consuming to configure and complicated to use. Secondly, the electrodes must be significantly smaller than the cells in size, to allow manipulation of the cells by pushing. As a result, the electrodes are again expensive, difficult to construct and extremely fragile, thereby further increasing the cost and complexity of the apparatus.

In addition to this, touching the cells with the electrodes can lead to additional problems, such as burning of the cells. Even in the event that a signal is not being applied to the electrodes when the cell is pushed, the electrodes can retain a residual field from when they are last used. In this case, contact of the cell with the electrode can cause the field to be discharged, thereby damaging the cell.

Finally, the use of the laser trapping and electrodes to manipulate cells is difficult to achieve manually as described in WO01/09297. This not only means that training is required to perform cell fusion using the apparatus, but also means the cell fusion process itself can be time consuming.

30

### **Summary of the Present Invention**

In a first broad form the present invention provides a method of fusing first and second cells, the method including:

- a) Selecting the first and second cells;
- 5      b) Positioning the first and second cells between two electrodes in a fluid filled fusing container, the first and second cells being held in suspension separated from each electrode; and,
- c) Applying a current having a predetermined waveform to the electrodes to cause the cells to fuse.

10

The method of selecting the first and second cells typically includes using a pipette to extract:

- a) The first cell from a group of first cells held in a first container; and,
- b) The second cell from a group of second cells held in a second container.

15

The method of positioning the first and second cells between the two electrodes usually includes:

- a) Using the pipette to position the first cell in the fusing container;
- b) Using the pipette to position the second cell in the fusing container, adjacent the first cell;
- 20      c) Positioning the electrodes such that the first and second cells are located substantially between the electrodes.

20

The pipette is typically coupled to:

- 25      a) A drive system adapted to move the pipette with respect to the first, second and fusing containers; and,
- b) An actuator adapted to actuate the pipette to thereby expel or draw in fluid through a port.

30      In this case, the method usually includes using a controller coupled to the drive system and the actuator to move and actuate the pipette.

- 5 -

The method of selecting a cell preferably includes causing the controller to:

- a) Move the pipette such that the port is adjacent a cell having predetermined characteristics, the cell being held in fluid suspension in the respective container;
- 5      b) Actuate the pipette to draw in fluid through the port, thereby drawing in the cell and the surrounding fluid.

The method of using the pipette to position the second cell adjacent the first cell generally includes causing the controller to:

- 10      a) Move the pipette such that the port is adjacent the first cell in the fusing container;
- b) Cause the pipette to expel fluid through the port, thereby expelling the second into the fluid in the fusing container;
- c) Move the pipette such that the port is as close as possible to both the first and second cells;
- 15      d) Cause the pipette to draw in fluid through the port, thereby drawing in the first and second cells and the surrounding fluid;
- e) Cause the pipette to expelling the first and second cells into the fluid in the fusing container; and,
- f) Repeat steps (c) to (e) until the first and second cells are within a predetermined
- 20      distance.

The electrodes may be coupled to an electrode drive system adapted to move the electrodes with respect to the fusing containers, in which case the method typically includes using a controller coupled to the electrode drive system to position the electrodes in the fusing  
25      chamber.

The electrodes may be coupled to a waveform generator, in which case the method of applying the alternating current includes causing the waveform generator to apply a predetermined waveform to the electrodes.

30

- 6 -

If the first and second cells having a respective cell type, the method preferably includes using a controller coupled to the field generator to select the waveform in accordance with the cell types of the first and second cells.

- 5 The first and second cells may be the same type of cell, the first and second group of cells being the same group.

In a second broad form the present invention provides apparatus for fusing first and second cells, the apparatus including:

- 10 a) A fluid filled fusing container;
- b) At least two electrodes adapted to be positioned in the fusing container in use;
- c) A selector for:
  - i) Selecting a first cell from a group of first cells held in a respective container;
  - and,
  - 15 ii) Selecting a second cell from a group of second cells held in a respective container;
  - iii) Positioning the first and second cells in the fusing container, the first and second cells being held in suspension; and,
- d) A field generator coupled to the electrodes, the field generator being adapted to  
20 cause a field having a predetermined waveform to be generated between the electrodes, thereby causing the cells to fuse.

The selector is preferably a pipette.

- 25 The apparatus generally further includes:
  - a) A drive system adapted to move the pipette with respect to the first, second and fusing containers; and,
  - b) An actuator adapted to cause the pipette to expel or draw in fluid through a port.
- 30 The electrodes may be coupled to the fusing container.



- 7 -

Alternatively the apparatus can include an electrode drive system adapted to move the electrodes with respect to the fusing containers.

The apparatus also typically includes a controller adapted to control the fusing of the cells  
5 by controlling operation of at least one of:

- a) The pipette;
- b) The electrodes; and,
- c) The field generator.

10 The controller typically includes a processor coupled to at least one of:

- a) The drive system and the actuator, the processor being adapted to move and actuate the pipette;
- b) The electrode drive system, the processor being adapted to move the electrodes; and,
- 15 c) The field generator, the processor being adapted to cause the field generator to generate the field having the predetermined waveform.

The controller may include a detector adapted to detect the position of cells within the containers, in which case the processor can be responsive to the detector to move at least  
20 one of the electrodes and the pipette in response to the position of detected cells.

Alternatively, or additionally, the processing system may include an input for receiving input commands from a user.

25 The processor can be coupled to a store for storing waveform data representing a number of different predetermined waveforms, the processor being adapted to select one of the number of predetermined waveforms in response to the input commands received from the user.

30 The processor can also being adapted to move at least one of the electrodes and the pipette in response to the input commands received from the user.

Typically the controller is adapted to cause the cells to fuse by causing the apparatus to perform the method of the first broad form of the invention.

5 In a third broad form the present invention provides, a controller for controlling apparatus for fusing first and second cells, the apparatus including:

- a) A fluid filled fusing container;
- b) At least two electrodes;
- c) A selector;
- 10 d) A field generator coupled to the electrodes;

Wherein, in use, the controller is adapted to cause the cells to fuse by:

- i) Causing the selector to:
  - (1) Select a first cell from a group of first cells held in a respective container;
  - and,
  - 15 (2) Select a second cell from a group of second cells held in a respective container; and,
  - (3) Position the first and second cells in the fusing container between the electrodes, the first and second cells being held in suspension;
- ii) Positioning the electrodes in the fusing container; and,
- 20 iii) Causing the field generator apply a field having a predetermined waveform to the electrodes, thereby causing the cells to fuse.

The controller can also be adapted to position the cells in the fusing container.

25 In this case, the controller typically includes a processor coupled to at least one of:

- a) A drive system adapted to move the pipette with respect to the first, second and fusing containers;
- b) An actuator adapted to cause the pipette to expel or draw in fluid through a port;
- c) An electrode drive system adapted to move the electrodes with respect to the fusing
- 30 containers; and,
- d) The field generator.

- 9 -

The controller is typically adapted to operate for use with apparatus of the second broad form of the invention.

- 5 In this case, the controller is preferably adapted to cause the apparatus to perform the method of the first broad form of the invention.

In a fourth broad form the present invention provides a computer program product for controlling apparatus for fusing first and second cells, the computer program product  
10 including computer executable code which when executed by a suitable processing system causes the processing system to operate as the controller of the third broad form of the present invention.

In a fifth broad form the present invention provides a pipette system for manipulating  
15 particles, the pipette system including:

- a) A nozzle for containing fluid in use, the nozzle including a port;
- b) An actuator coupled to the nozzle, the actuator being adapted to draw in and/or expel fluid through the port; and,
- c) An electrode coupled to the nozzle adjacent the port, the electrode being adapted to  
20 cooperate with a second electrode to allow an electric field to be applied to coupled to one or more particles positioned adjacent the port.

The electrode is usually formed a conductive tube.

- 25 The electrode may be formed from a stainless steel tube having a diameter of approximately 10mm.

The pipette system can include a drive system adapted to move the pipette system to be with respect to a fluid filled container to thereby allow particles to be positioned in or  
30 removed from fluid in the container.

- 10 -

The pipette system can include a field generator coupled to the electrode for generating a predetermined electric field between the electrode and a second electrode positioned in the container.

- 5 The pipette system typically includes a controller adapted to control the drive system, the actuator and the field generator to thereby apply an electric field to a particle by:
  - a) Positioning the particle in the container adjacent the second electrode using the pipette;
  - b) Positioning the pipette port adjacent the particle in the container; and,
  - 10 c) Activating the field generator.

The controller is typically adapted to fuse cells, by:

- a) Positioning a first cell in the container adjacent the second electrode using the pipette;
- 15 b) Positioning a second cell in the container adjacent the first cell using the pipette;
- c) Positioning the pipette port adjacent the first and second cells, such that first and second cells are substantially between the electrodes; and,
- d) Activating the field generator to cause a predetermined field sequence to be applied to the cells, thereby causing the cells to fuse.

20

The pipette system generally further includes:

- a) A radiation source; and,
- b) A waveguide having a first end coupled to the radiation source and a second end coupled to the nozzle adjacent the port to thereby allow radiation from the radiation source to impinge on particles positioned adjacent to the port in use.

25

The pipette system can include a detector, the detector being adapted to detect radiation emitted by the particle.

- 30 The detector can be coupled to the first end of the waveguide, to thereby detect radiation emitted from the particle.

The radiation is typically a laser, although other sources, such as LEDs may be used.

5 The waveguide can be a fibre optic cable, or alternatively can being formed from the nozzle, the nozzle including a shaped portion to allow the radiation from the radiation source to enter the nozzle and pass along at least a portion of the nozzle, the radiation being emitted from the nozzle through the port.

The pipette system generally includes a controller adapted to perform at least one of:

- 10 a) Activating the actuator to thereby cause fluid to be drawn in and/or expelled through the port; and,  
b) Activating the radiation source, to thereby expose a particle to radiation.

15 The drive system can be coupled to a controller, the controller being adapted to recover particles having predetermined properties from the container by:

- a) Positioning the pipette system such that the port is adjacent to a particle;  
b) Activating the radiation source to thereby expose the particle to radiation;  
c) Detect any radiation emitted by the particle;  
d) Determine if the particle has the predetermined properties in accordance with the  
20 detected radiation; and,  
e) In accordance with a successful comparison, activate the actuator to thereby draw fluid into the nozzle through the port, thereby recovering the particle.

The actuator can include:

- 25 a) A fluid reservoir;  
b) A flexible tube coupling the nozzle to the fluid reservoir;  
c) An arm positioned so as to partially compress the tube;  
d) An actuator drive system adapted to move the arm so as to perform at least one of:  
i) Further compressing the tube to thereby expel fluid from the port; and,  
30 ii) Decompressing the tube to thereby draw fluid in through the port.

- 12 -

The actuator drive system generally includes:

- a) A first actuator drive for moving the arm with respect to the tube; and,
- b) A second actuator drive formed from an arm end portion, the arm end portion being in contact with the tube in use, the second actuator drive being adapted to cause the arm end portion to expand or contract.

5

The pipette system usually includes a controller coupled to the actuator drive system, the controller being adapted to operate the actuator drive system to thereby draw fluid in or expel fluid through the port.

10

The drive system can be coupled to the controller, the controller being adapted to recover particles from the fluid by:

- a) Positioning the pipette system such that the port is adjacent to a particle; and,
- b) Activate the actuator drive system to thereby draw fluid into the nozzle through the port, thereby recovering the particle.

15

The tube can be formed from silicon tubing.

#### **Brief Description of the Drawings**

- 20 An example of the present invention will now be described with reference to the accompanying drawings, in which: -

Figure 1 is a block diagram of an example of apparatus for implementing the present invention;

- 25 Figure 2 is a schematic diagram of the apparatus of Figure 1;

Figure 3 is a schematic diagram of the pipette of Figure 1;

Figure 4 is a flow chart of an overview of the process implemented by the apparatus of Figure 1;

- 30 Figures 5A to 5C are a flow chart of an overview of the process implemented by the apparatus of Figure 1;

Figures 6A and 6B are schematic diagrams of cells being drawn into and expelled from the

- 13 -

pipette of Figure 3;

Figures 6C and 6D are schematic diagrams of the arrangement of the electrodes and cells in the fusion well during operation of the apparatus of Figure 1; Figures 7A to 7G are examples of pulse sequences that may be used in the apparatus of Figure 1;

- 5 Figure 8 is a schematic plan view of the well array of Figure 2 modified to include electrodes;

Figure 9 is a schematic side view of the modified well array of Figure 8;

Figure 10 is a schematic diagram of the pipette of Figure 3 modified to include an electrode;

- 10 Figure 11 is a block diagram of a modified version of the apparatus of Figure 1 adapted to use two of the pipettes shown in Figure 10;

Figure 12 is a schematic diagram of the apparatus of Figure 11;

Figure 13 is a schematic diagram of the pipette of Figure 10 modified to include a radiation source;

- 15 Figure 14 is a schematic diagram of the pipette of Figure 3 retrieving a number of cells;

Figure 15 is a schematic diagram of the pipettes of Figure 11 positioning cells for subsequent fusion;

Figure 16 is a schematic diagram of the pipettes of Figure 11 and fused cells;

Figure 17A is a schematic diagram of the pipette of Figure 3 with an alternative actuator;

- 20 and,

Figure 17B is a schematic diagram of the operation of the actuator of Figure 17A.

### **Detailed Description of the Preferred Embodiments**

An example for apparatus suitable for implementing the present invention will now be  
25 described with reference to Figures 1, 2 and 3.

As shown in Figure 1, the apparatus includes a processing system 10 coupled to an imaging system 11, a control system 12 and a signal generator 13. The control system 12 is coupled to a pipette system 14 and an electrode system 15, as shown.

30

The processing system 10 includes a processor 20, a memory 21, an input/output (I/O)

- 14 -

device 22, an image interface 23, a control interface 24, and a signal interface 25, coupled together via a bus 26. The processing system may therefore be any one of a number of systems, such as a suitably programmed computer, specialised hardware, or the like. In any event, the I/O device typically includes a display, such as a computer monitor or the like, a keyboard, and one or more other input devices such as a mouse, joystick, trackball or the like.

The imaging system 11 includes a camera 30 such a CCD camera or the like which is coupled to a microscope 31. The imaging system 11 is connected to the processing system via the image interface 23.

The pipette system 14 includes a pipette shown generally at 33 that is coupled to the control system 12 via a drive system 32. In use, the control system 12 is coupled to the processor via the control interface 24, thereby allowing the drive system 32 to be used to control motion and operation of the pipette, as will be described in more detail below.

Similarly, the electrode system 15 is formed from two electrodes 35 coupled to the control system 12 via a drive system 34. Again, the control system 12 allows the drive system 34 to control the position of the electrodes, as will be described in more detail below.

In use, the system allows a user to select and move individual cells using the pipette system 14. When appropriate cells are placed next to each other, this allows an electric field to be applied to the cells using the electrodes 35 thereby causing the cells to fuse.

In order to achieve this, the apparatus is arranged as shown schematically in Figure 2 such that the pipette 33 and the electrodes 35 may be moved relative to a well array shown generally at 40. This allows cells to be moved between the wells 40, 41, 42, 43, 44, 45, 46, 47, 48, as shown.

Movement of the pipette and the electrodes 35 is achieved by operation of the corresponding drive system 32, 34. Accordingly, it will be appreciated that the processing



- 15 -

system 10 may be used to control positioning of the pipette 33 and the electrodes 35 allowing the pipette 33 and the electrodes 35 to be inserted into and positioned within a respective one of the wells 41, ..., 48.

- 5 Furthermore, the microscope 31 is arranged to image selected wells 41, ..., 48 such that the representation of the contents of a selected well can be displayed to the user using the I/O device 22.

In general, the processing system 10 is adapted to control the pipette 33 and the electrodes  
10 35 in accordance with input commands received from the user via the I/O device 22. In order to achieve this, the processing system 10 must be able to perform a number of functions simultaneously, such as:

- Presenting an image of the well array 40 to the user on the I/O device 22;
- 15 • Responding to commands input via the I/O device 22 to move and, if required, actuate the pipette system 14;
- Responding to command inputs via the I/O device 22 to move the electrodes 35; and,
- Responding to commands input via the I/O device 22 to apply an electrical signal to the electrodes 35.

20

This is achieved by having the processor 20 execute appropriate application software which is stored in the memory 21.

The pipette is shown in more detail in Figure 3. As shown, the pipette 33 is formed from a  
25 housing 50 defining a chamber that is divided into two portions 51A, 51B by a piezo-electric element 52, as shown. The chamber 51B is coupled by a port 53 to a flexible tube 54. The flexible tube 54 includes a male coupling 55 that is adapted to cooperate with a female coupling 56 positioned on a shaped glass nozzle 57 having an aperture 58, as shown.

30

In use, the chamber 51B, the port 53, the flexible tube 54 and the glass nozzle 57 are filled

- 16 -

with fluid, with the chamber 51A being filled with air and sealed. Applying a current to the piezo-electric element 52, via leads 59, causes the element to move, with the direction of movement depending on the polarity of the applied current.

5 Thus, in use, with the aperture 58 positioned in fluid in one of the wells 41, ..., 48, causing the piezo-electric element 52 to move in the direction of the arrow 60 will increase the volume of the chamber 51B, thereby causing fluid to be drawn through the aperture 59. Similarly, causing the piezo-electric element 52 to move in the direction of arrow 61 will decrease the volume of the chamber 51B, thereby causing fluid to be expelled through the  
10 aperture 58.

Accordingly, the pipette can be activated to draw in or expel fluid through the aperture 58 depending on the polarity of the current applied to the leads 56. Accordingly, in use, the leads 56 are coupled to either the drive system 32, or a separate activation system, to allow  
15 a suitable current to activate the pipette as required.

The manner in which the apparatus is used to fuse cells will now be described.

#### Overview

20 An overview of the method of fusing cells in accordance with the present invention will now be described with reference to Figure 4.

In particular, at step 100, the user selects the cells to be fused. At step 110, the cells are placed in a fusion well.  
25

At step 120 a predetermined electric field is applied to the selected cells to cause the cells to fuse.

Cells that are placed in an electric field will distort the field in their immediate vicinity.  
30 The field distortion is dependent on the geometry and electrical properties of the particle and that of the surrounding particles. Living cells have interior (cytoplasm) that is highly

- 17 -

conductive, due to the accumulation of ions such as potassium (K<sup>+</sup>) ions, and a relatively high dielectric constant. The membrane surrounding has a very low conductivity and a lower dielectric constant.

5 Accordingly, the degree of the distortion of the field both inside and outside of the cell is a very strong function of the frequency of the applied electric field. As a result when placed in a non-uniform electric field cells will experience a force whose magnitude and direction will vary in a complicated manner with the frequency of the applied field. This effect can be exploited to selectively manipulate living cells using radio-frequency alternating  
10 electric fields created via suitable electrodes. The movement of particles in AC electric fields is referred to as 'dielectrophoresis' (DEP) and is independent of any net charge on the particle.

The application of the radio-frequency electric fields exerts a positive DEP force on the  
15 two cells, urging the cells into close contact with each other. A stronger electric field is then used in order to induce electrical breakdown of each cell's membranes at their point of contact. This controlled electro-poration triggers a process of cell fusion that is somewhat akin to reverse-mitosis. This in turn creates a fused hybrid cell that has a genetic makeup that is a combination of the two original cells that were fused.

20

The fused cell is then generally placed in a recovery well at step 130 before being checked after a predetermined time period to confirm the cell has fused at step 140.

The fused cells can then be collected at 150 and used as required.

25

#### **Detailed Description**

A detailed example of the method of using the apparatus of the present invention will now be described with reference to Figures 5A, 5B, 5C and 5D.

30 In this example, the well array 40 includes a target well 41, a partner well 42, a washing well 43, a fusion well 44, a recovery well 45 and a hybrid well 46 the purpose of which

- 18 -

will be described in more detail below.

At step 200 the target and partner cells are placed in respective target and partner wells. This procedure will generally involve suitable preparation of the cells, which may be  
 5 achieved in a number of manners. Thus, for example, this may require that the cells are recovered from sample plates and washed in appropriate enzyme solutions.

The well array would then be sterilised before appropriate fluids are inserted into the wells to be used. The target and partner cells are then placed in the target and partner wells, 41,  
 10 42 respectively, with the cells being held in suspension in respective enzyme solutions.

At step 210, the user selects a target cell from the target well 41 using the pipette 33. In order to achieve this, the user will arrange the well array 40 such that the target well 41 is imaged by the imaging system. Accordingly, the target well 41 is placed under the  
 15 microscope 31 so that the camera 30 may generate an image signal and transfer this to the image interface 23. The image signal will then generally undergo some pre-processing in the image interface 23 before being transferred to the processor 20 for any subsequent further processing.

20 Thus, for example, the image interface 23 may be formed from an image capture card, which is used to capture images from incoming image signals. The captured image is then formatted by the processor 20 before being presented to the user using the I/O device 22.

The user adjusts the relative position of the microscope 31 and the well array until a  
 25 suitable target cell is shown. The user then uses the processing system 10 to control the position of the pipette 33. In particular, this is usually achieved by having a joystick I/O device 22, with the processor 20 responding to signals from the joystick to generate commands which are transferred via the control interface 21 to the control system 12. The control system will typically be formed from a motion control addressing amplifier, which  
 30 is coupled to a drive system 32, such as suitable stepper or DC servo motors.

- 19 -

By use of appropriate sensitivity control, this allows the position of the pipette to be controlled to high degree of accuracy. By arranging the microscope such that the pipette is shown in the image presented on the display, this allows the user to position the pipette 33 with the pipette aperture 58 adjacent the selected cell.

5

At this point, the user activates the pipette 33 to draw fluid in through the aperture 58. The cell and the surrounding fluid will be drawn into the pipette, allowing the target cell to be removed from the target well 41.

10 Sometimes, it is difficult to separate individual cells within the wells. This can be overcome by repeatedly operating the pipette to cause the pipette to repeatedly draw in and expel fluid via the pipette aperture 58. Agitation of the fluid medium and repeated movement of the cells through the pipette aperture 58 will usually allow a cell to be separated from surrounding cells.

15

An example of this is shown in Figure 6A, which shows the hydrodynamic stream-lines 70 as fluid is expelled from the pipette aperture 58. As shown, the hydrodynamic stream-lines, which represent lines of constant force, spread out away from the pipette aperture 58. Similarly, as the cells, shown at 71, 72, are entrained in the fluid flow, this will tend to  
20 cause the cells 71, 72 to separate as they are expelled away from the pipette aperture 58.

In any event, once the user has selected the target cell at step 210, the user washes the target cell in a fusion medium in the washing well 43. In order to do this, the pipette containing the respective cell is positioned in the washing well 43, using the imaging and  
25 control system 11, 12 to move the pipette 33 as described above. Once the pipette 33 is positioned inside the washing well 43, the pipette is repeatedly activated to cause fluid to be drawn in through and expelled through the pipette aperture 58. In this way, the cell is repeatedly placed in the fusion medium in a washing well 43 and then removed. This action causes the cell to be washed.

30

Furthermore, when the user transfers the target cell to the fusion well 44 at step 230,

- 20 -

this is achieved by positioning the pipette 33 in the washing well 43 and drawing the target and cell into the pipette 33 through the pipette aperture 58. Accordingly, at this point the target cell is surrounded in fusion medium as opposed to in the medium contained in the target well 41.

5

The user then uses the pipette 33 to place the target cell into the fusion well 44 at step 230. Steps 210 to 230 are repeated for the partner cell, with the partner cell being placed in the fusion well 44 next to the target cell at 230.

- 10 As an alternative to performing steps 210 to 230 separately for each cell, the target and partner cells may be selected from the respective wells and then washed together in the washing cell 43 being transferred simultaneously to the fusion well 44.

- 15 As will be described in more detail below, it is preferable for the cells 71, 72 to be positioned adjacent to each other. In order to achieve this, it is preferable to first place the target or partner cell 71 in the fusion well 44 and then place the other partner or target cell 72 adjacent thereto.

- 20 In general as adding the second cell 72 will cause fluid to be transferred into the fusion well 44, this also causes movement of the first cell 71. It is then generally necessary to repeatedly activate the pipette 33 until the both cells can be drawn in to the pipette simultaneously. As shown in Figure 6B, when the cells 71, 72 are drawn in to the pipette aperture simultaneously, the hydrodynamic lines of force 70 converge as the fluid enters the aperture 58. Accordingly, this draws the cells 71, 72 together. The cells can then be  
25 expelled from the pipette 33 with the cells being sufficiently close for the fusion process to be performed.

- In any event, once the user has positioned the target and partner cells in the fusion well at 230 the user then arranges to place the electrodes 35 in the fusion cell 44 at step 240.  
30 Again, in order to achieve this, the imaging system 11 is positioned such that the I/O device 22 presents the user with an image of the fusion cell 44.

The user can then alter the position of the electrodes 35 by providing appropriate commands via the I/O device 22. Again, this is usually achieved by having a respective joystick or the like provide control signals to the processor 20. The processor then  
5 transfers appropriate command signals via the control interface 24 to the control system 12. The control system then activates the drive system 34, thereby causing the electrodes 35 to move as directed by the user.

An example of the relative positioning of the electrodes 35, the cells 71, 72 and the pipette  
10 33 at this stage is shown in Figures 6C and 6D, which show a perspective and end on view of the fusion well 44 prior to fusion being performed. Thus, as shown, the cells 71, 72 are positioned close to each other substantially between the electrodes 35. At this stage the cells need not be in contact as they will in any event be urged together by the applied electrical field as will be described in more detail below.

15

As shown in Figure 5B, the next step is for the user to determine the sequence of electric fields that are to be applied to the cells at step 260 before using the processing system 10 and the signal generator 13 to generate the determined pulse sequence at step 270.

20 The manner in which the user determines the electric field will vary depending on the particular implementation of the invention. A first example by which this may be achieved is shown in steps 280, 290. In this case, the processing system 10 applies a predetermined electric field to the partner and target cells. The response of the cells in the electric field is then used to determine the electrical parameters employed for the DEP electric field (in  
25 order to bring the cells together). The response can also be used to determine the fusion pulse sequence (including the frequency and amplitude) required to fuse any particular pair of cells. In particular, the processing system 10 will apply a field having a predetermined frequency. The frequency can then be fine adjusted until an optimum frequency is determined at which the force that attracts the cells to cells move toward each other is  
30 optimal for the required conditions. This response of the cells to the DEP electric field will occur due to the generation of electric dipoles within the cells, as described above.

- 22 -

The response of the cells to the electric field can be monitored either automatically by having the processor 20 execute appropriate image recognition software, or manually by the user. The processor would then select a pulse sequence from a number of pulse sequences stored in the memory 32. The pre-programmed pulse sequences would be stored in a look up table (LUT), or the like, in accordance with the field applied to obtain the desired response. It will be appreciated that this information may need to be determined initially. Accordingly, each time a new lineage of target and partner cell combination is fused, the pulse sequence used to achieve this successfully will be stored in the LUT and the memory 21, together with information regarding the complete set of fusion parameters at which the desired response was observed. The processor 20 can then use the indication of the response to select a pulse sequence from the LUT.

Alternatively, the pre-programmed pulse sequences could be stored in the LUT in accordance with each particular type of target and partner cell combination. Again, this information will need to be determined initially. However, by storing the pulse sequence each time a new target and partner cell combination is fused, this allows the processor 20 to select a pulse sequence at step 310 in accordance with cell types provided by the user at step 300.

In any event, the electric pulse sequences applied to the cells to cause the cells to fuse by DEP at step 320.

At step 330, whilst the cells are still in the fusion well 44 the user examines the both morphology and the electrical behaviour of the cells to determine if they have fused to create a fusate cell. If the morphology and behaviour appear favourable to fusion then the fusate is transferred using the pipette 33 to the recovery well 45 at step 360. The initial stages of cell fusion only take a few minutes, typically under ten for most type of cells and accordingly, the user can simply view the cells on the I/O device 22 and determine from this whether the fusion process has been successful. If it is determined that the cells have not fused at step 340, the user simply discards the unfused cells with the pipette 33 at step



- 23 -

350, and returns to select new cells at step 210.

Once placed in the recovery well 45 the fusate cell is left for approximately 45 minutes before again being checked at step 370. During this time, the cell is held in suspension in a  
 5 suitable culture medium to encourage cell growth. If it is determined that the fusate cell has not completely fused at step 380 then the user discards the unfused cells using the pipette 33 at step 390, and selects new cells at step 210.

Otherwise, the user transfers the fusate cell to a respective hybrid well 46 using the pipette  
 10 33 at step 400. The fusate cell is incubated in the hybrid well at step 410, with the cell being monitored after and during the incubation process at step 420, to determine if the fusion has been successful.

#### **Pulse Sequences**

15 As described briefly above, different pulse sequences may be used to control the fusion of the two cells. The generation of different pulse sequences is achieved by having the processor 20 control the signal generator 13 in accordance with pre-determined pulse sequences stored in the memory 21. The pulse sequences are generally stored in data  
 20 arrays and associated parameters in an LUT, as outlined above or calculated using suitable equations and data arrays at the point of fusion. The processor 20 extracts the necessary parameters and the like stored in the memory 21 and transfers this information to the signal interface 25.

In this example, the signal interface 25 is in the form of an arbitrary waveform generator or  
 25 the like, which uses the determined parameters to define a desired pulse sequence. The waveform generator therefore generates a signal representative of the pulse sequence and transfers this to a high frequency signal amplifier, allowing the desired pulse sequence to be transferred to the electrodes 35 as required.

30 It will be appreciated that other forms of pulse sequence generation can also be used.

- 24 -

In any event, an example of different electrical pulse sequences that may be used for fusing cells will now be described. In each of these examples, the functions are defined in the temporal domain,  $t$

5 The basic pulse sequence profiles may be defined in terms of the equations:

$$\begin{aligned} y_1(t) &= A \sin(\omega t) &< t_1 \\ y_2(t) &= C(t) &t_1 < t < t_2 \\ y_3(t) &= B \sin(\omega t) &> t_2 \end{aligned}$$

where: A is a constant

B is a constant (and may be equal to A)

10  $C(t)$  is the function describing the pulse.

$C(t)$  is typically based on one of the following functions, although it will be appreciated that this is not essential:

$$\begin{aligned} C_1(t) &= \pm K \\ C_2(t) &= Q \exp(-\alpha t) \\ C_3(t) &= Q \exp(\alpha t) \\ C_4(t) &= Q \sin(\xi t) \end{aligned}$$

15

where:  $K$ ,  $Q$ ,  $\alpha$  and  $\xi$  are constants.

Basic pulse sequences can be combined and overlaid to create complex sequences, some examples of which are listed below and are shown in Figures 8A to 8G.

20

Figure 7A shows a first example of a Basic DC Fusion Pulse Sequence consisting of 2 unipolar square pulses, separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

- 25 -

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K &t_1 < t < t_2 \\
 y_3(t) &= B \sin(\omega t) &t_2 < t < t_3 \\
 y_4(t) &= +K &t_3 < t < t_4 \\
 y_5(t) &= A \sin(\omega t) &> t_4
 \end{aligned}$$

Figure 7B shows a second example of a Basic DC Fusion Pulse Sequence consisting of a bipolar square pulse, separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K &t_1 < t < t_2 \\
 y_3(t) &= -K &t_2 < t < t_3 \\
 y_4(t) &= A \sin(\omega t) &> t_3
 \end{aligned}$$

Figure 7C shows a third example of a Basic AC Fusion pulse consisting of a sinusoidal (of differing frequency) of increased amplitude and differing frequency separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= Q \sin(\xi t) &t_1 < t < t_2 \\
 y_3(t) &= B \sin(\omega t) &t_2 < t < t_3 \\
 y_4(t) &= Q \sin(\xi t) &t_3 < t < t_4 \\
 y_5(t) &= A \sin(\omega t) &> t_4
 \end{aligned}$$

Figure 7D shows a fourth example of a Basic DC and exponential pulse separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K &t_1 < t < t_2 \\
 y_3(t) &= K + Q \exp(-\alpha t) &t_2 < t < t_3 \\
 y_4(t) &= A \sin(\omega t) &> t_3
 \end{aligned}$$

Figure 7E shows a fifth example of a Basic DC and exponential pulse separated by sinusoidal waves. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K &t_1 < t < t_2 \\
 y_3(t) &= K - Q \exp(\alpha t) &t_2 < t < t_3 \\
 y_4(t) &= A \sin(\omega t) &> t_3
 \end{aligned}$$

Figure 7F shows a sixth example of a Basic DC pulse sequence convoluted with a linear curve. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K \otimes (-\beta t) &t_1 < t < t_2 \\
 y_3(t) &= B \sin(\omega t) &t_2 < t < t_3 \\
 y_4(t) &= +K \otimes (-\beta t) &t_3 < t < t_4 \\
 y_5(t) &= A \sin(\omega t) &> t_4
 \end{aligned}$$

Note. An extra DC pulse is shown in Figure 7F for clarity.

Figure 7G shows a seventh example of a Basic DC pulse convoluted with an exponential decay curve. The equations used to govern the generation of these pulse sequences are as follows:

$$\begin{aligned}
 y_1(t) &= A \sin(\omega t) &< t_1 \\
 y_2(t) &= +K \otimes (Q \exp(-\alpha t)) &t_1 < t < t_2 \\
 y_3(t) &= B \sin(\omega t) &t_2 < t < t_3 \\
 y_4(t) &= +K \otimes (Q \exp(-\alpha t)) &t_3 < t < t_4 \\
 y_5(t) &= A \sin(\omega t) &> t_4
 \end{aligned}$$

Note. An extra DC pulse is shown in Figure 7G for clarity.

**Specific Example**

An outline of the production of a human-human hybridoma using the apparatus of Figure 1 will now be described. In general the explanation will focus on the following staged of the process.

- 5     • Preparation of the cells for fusion
- Setup of the apparatus for fusion
- Manipulation of the cells in readiness for fusion
- Electrofusion of the selected pair of cells to obtain hybrid fusates.

10    Preparation of the cells for fusion.

Peripheral Blood Mononuclear Cells (PBMC) were prepared according to the following protocol: Buffy Coats are obtained from healthy donors (sero-negative for HIV, Hep-B, Hep-C, HTLV-I and Syphilis) from the Australian Red Cross Blood Bank, Sydney, NSW. PBMC are isolated by density centrifugation on Ficoll-Paque<sup>TM</sup> Plus (Amersham Pharmacia, 17-1440-03). The B cells are then isolated for fusion. Untouched B cells are isolated from PBMC with MACS B Cell Isolation Kit (Miltenyi BioTec, 469-01) by magnetic depletion of T cells, NK cells, myeloid cells, basophils, platelets and early erythroid cells. A human myeloma cell line, designated F4 was used as the immortal partner cell.

20

Set-up of the apparatus for fusion

- A 20ml syringe (#1) was loaded with RPMI media (#2) warmed in an incubator (30 mins at 37 C). Using the syringe a large droplet of the warmed RPMI solution was deposited into the centre of a Petrie dish (#3). This dish was then placed on the inverted microscope (Nikon TE2000) such that it was situated beneath the pipette. The pipette having first been sterilized with repeated washings of 70% alcohol/water solution. The pipette was then lowered so that the tip was immersed in the droplet of RPMI. One end of a length of silicon tubing(#4) (with suitable connectors(#5)) was attached to a second syringe and the other end to the pipette. RPMI was then gently drawn into the pipette and through the tubing using the syringe. Care was taken to ensure that no air bubbles formed anywhere along the tubing or in the pipette. Using the RPMI filled syringe, fluid was injected into
- 25
- 30

- 28 -

the nozzle of the piezo electric actuator until it was completely filled and a positive meniscus formed over the nozzle. The second syringe was then gently uncoupled from the silicon tubing. Using the first syringe filled with RPMI the uncoupled end of the silicon tubing was topped with fluid until a positive meniscus over the mouth of the connector. The tubing was then coupled to the piezo electric nozzle 54.

Each pipette nozzle 54 is drawn from capillary tubing (120µm inner diameter) from (#7)

The electrodes 35 were then aligned using a graticule until they were spaced ~400 – 500 µm apart.

The previously prepared partner cells were then transferred to a single well in a 96 well plate (#6) and the lymphocytes were deposited in a separate well in the same plate.

The pipette was then inserted into the well containing the partner cells and a suitable partner cell selected. This (single) cell was then transferred to a fresh well containing RPMI + 10% fetal calf serum; FCS. The pipette was then inserted into a well containing the previously sorted B lymphocytes specific to the target antigen. A suitable B lymphocyte for fusion was then selected. Returning to the previous well the lymphocyte cell was expelled from the pipette beside the partner cell. Both cells were then visually inspected for their suitability for fusion.

#### Manipulation of the cells prior to fusion

The pipette was then used to transfer both cells into a well containing a sorbitol solution of appropriate pH and osmolarity. The cells were then 'washed' in the fusion media by gently inhaling and expelling them through the pipette aperture 58 in order to allow them to acclimatise to the changed environment. Once the cells had adjusted to the change in osmolarity the pipette was then used to hydro-dynamically arrange the cells so that they were within 5-10µm of each other. The pipette was then removed from the well.

- 29 -

The electrodes 35 were inserted into the well and arranged so that the previously arranged cells lay centred and co-linearly between them. Each electrode is constructed from a nickel alloy wire of 180 $\mu$ m diameter manufactured by the Californian Fine Wire Company, California, USA. The configuration of the electrodes, their shape and their proximity to the cells are specifically designed so that a suitable electric field pattern can be generated in order to induce DEP between the cells.

The electrodes were connected through an amplifier to the arbitrary waveform generator and a series of voltages conforming to different waveforms, previously defined by the user, were applied. The first waveform applied to the electrodes was sinusoidal and had a frequency of 500 kilohertz and an amplitude, post amplifier, of approximately 6V peak to peak. Through phenomena known as dielectrophoresis, whereby neutral particles become polarised in the presence of an alternating, non-uniform, electric field, the cells experienced a force of attraction that caused them to coalesce.

The amplitude of the field was then increased to 15V peak to peak for a period of 5 seconds ensure that good membrane contact was made between the cells. In this increased field there was a slight drift of the cells towards the upper electrodes, and to counter this the stage of the microscope was adjusted relative to the electrodes to correct and retain the cells position between the electrodes.

#### Electrofusion of the selected pair of cells to obtain hybrid fusates.

Once the cells were suitably arranged a field pattern conforming to the DC pulse sequence was applied. In this instance the DC pulse sequence consisted of two pulse trains, each train consisting of 2 DC pulses, of amplitude 90V, (resulting in an electric field of approximately 180kV) each being of 80 $\mu$ s duration. The pulses were separated by a duration of 100 $\mu$ s, and each train was separated by 500 milliseconds, during which in the intervening time a DEP was applied in order to keep the cells in good contact. Post fusion pulse sequence, an increased DEP field was applied in order to maintain good contact between the cells whilst the cells fused.

- 30 -

#### Recovery of the cells to growth medium

The electrodes 35 were retracted from the fusion well, and the pipette 33 was inserted and manipulated so that the newly created fused cells were in the vicinity of the pipette aperture 58. The cells were then inhaled into the pipette and the pipette retracted from the well. In this fashion the cells were transferred to a fresh well containing hybridoma growth media (RPMI + 10% FCS). The newly fused cells were the only cells that were present in this media.

#### Automation

10 The above description focuses on manual use of the apparatus, in which positioning of the cells, electrodes and pipette are controlled in accordance with commands input by the user.

However, alternatively the processing system 10 can be adapted to control the apparatus automatically. In order to achieve this, the processor 20 executes image recognition applications software stored in the memory 21. This allows the processing system to use images received from the imaging system 11 to determine the position of cells within the wells 41, ... 48, as well as to determine the position of the electrodes 33 and the pipette 33.

From this, it will be appreciated that the processor 20 can be programmed to perform the procedure outlined above automatically. Accordingly, the processing system will be adapted to automatically select target and partner cells in accordance with the appearance of the cell in the image. The cells will then be placed in the fusion well 44 to allow the fusion to be performed. Again, during this process the processor 20 will control the position of the cells and the electrodes.

25

The processor then determines the pulse sequence to be applied to the cells, and applies the pulse sequence via the electrodes 35. Once this is completed the processor 20 can monitor the cells to determine if the fusion process is successful.

30 It will be appreciated that this may advantageously be achieved using the apparatus described in our copending application entitled "Cell Recovery".



### Modifications

A second example of apparatus suitable for implementing the present invention will now be described with reference to Figures 8 and 9.

5

In particular, the apparatus is substantially the same as the apparatus described above with respect to Figures 1 to 3. However, in this example, the apparatus includes a modified well array 40 having electrodes incorporated therein. Accordingly, the electrodes 35 are not required with the electrode system 15 utilising the electrodes within the well array as will be described in more detail below.

10

An example of the modified well array is shown in Figure 8. As shown, the well array 80 includes a fusion well 81. Mounted within the fusion well 81 are a number of pairs of electrodes 82A, 82B, 83A, 83B, 84A, 84B, 85, 85B. Although only four pairs of electrodes have been shown in this example, it will be appreciated that a greater number of electrodes may be used if an appropriately sized fusion well is provided.

15

The electrodes are typically formed from gold plated to a thickness of  $\sim 2\mu\text{m}$  onto a lower surface 86, as shown in Figure 9. The well array may also provided with one or more recovery wells 87, 88 as shown.

20

In use, the predetermined pulse sequences may be applied to the cells 71, 72 to be fused using the electrode pairs to 82, 83, 84, 85 as shown.

25

In use, the user will select the cells 71, 72 to be fused and position the cells between a respective pair of electrodes 82 using the pipette, as described above. Once the cells 71, 72 are positioned between the electrodes 82, the predetermined pulse sequence may be applied to the electrodes to thereby cause the cells to fuse in the manner described above.

30

From this it will be appreciated that four pairs of cells may be positioned in the fusion well 81 at any one time, as shown by the dotted lines. Whilst it is possible to fuse the four pairs

- 32 -

of cells simultaneously, it is possible for the field sequence generated each pair of electrodes 82, 83, 84, 85 to interfere. Accordingly, in some cases it is preferable for each pair of cells to be fused in sequence.

5 In order to achieve this, the processing system 10 can be adapted to apply a first predetermined pulse sequence to the electrodes 82, followed by a second predetermined pulse sequence to the electrodes 83, etc. It will therefore be appreciated that different field sequences may be applied to different pairs of electrodes to allow different cells to be fused within the same recovery well.

10

A third example of the present invention will now be described with reference to Figures 10 to 12.

15 In this example, apparatus similar to that in Figures 1 to 3 is again used with one of the electrodes 35 being replaced by an electrode provided on the pipette 33. An example of the pipette is shown in Figure 10.

As shown, the pipette is modified by the inclusion of an electrode 90 formed from a cylindrical tube 91, and which is coupled to the nozzle 57. The electrode 90 is coupled to  
20 the nozzle 57 such that the aperture 58 is contained in the tube 91 as shown.

In use, the pipette may be used substantially as described above to draw in an expel fluid through the port. This can be used to recover cells from a well allowing the cells to be placed in a fusion well, as described above.

25

In this example, the fusion well will additionally contain a second electrode. The second electrode may be a separate electrode similar to one of the electrodes 35 shown in Figure 2. The cells can then be positioned between the electrode 90 and the electrode 35. The signal generator is used to apply a predetermined pulse sequence to the electrodes 90, 35,  
30 allowing the cells to be fused as described above.

- 33 -

Alternatively, the electrode may be provided on the underside of the fusion well, in a manner similar to that shown in Figures 8 and 9.

As a third option, a second pipette 33B may be provided with a respective electrode 90B.

5 The resulting apparatus configuration is as shown in Figures 11 and 12, with the pipette system 14 being formed from two drive systems 32A, 32B and two pipettes 33A, 33B, as shown. Accordingly, in this example, electrodes 90A, 90B provided on the pipettes 33A, 33B, form the electrode system 15.

10 In any event, an electric field can be generated between the two electrodes 90A, 90B to allow cells 71, 72 to be fused in the manner described above.

It will be appreciated that the provision of a second pipette provides a number of additional advantages.

15

In particular, each pipette 33A, 33B is used to position respective cells 71, 72 adjacent each other by positioning the first cell 71 using the first pipette 33A, and then positioning the second cell 72 using the second pipette 33B. Once the cells are appropriately positioned, a pulse sequence can be generated between the two electrodes 90A, 90B, thereby causing the cells to fuse.

20

A number of additional developments can also be implemented for the pipettes. These include the provision of radiation sources such as lasers, LEDs, or the like, and appropriate detectors.

25

An example of this is shown in Figure 13. As shown, the pipette 33 includes an LED 92, arranged to direct radiation along the nozzle 57 and through the aperture 58, and electrode 90, as shown. The LED is typically coupled to the processing system 10, via leads 93, to allow the processing system to selectively activate the electrode as required. This allows a cell 71 adjacent the aperture to be exposed to radiation.

30

- 34 -

This allows cells to be labelled with fluorescent markers or the like, to allow the detectors to detect the cells having predetermined properties as described for example in our co-pending Patent Application entitled "Cell Recovery".

- 5 In addition to this, in the example of the system shown in Figure 12, each pipette 33A, 33B could be provided with an LED 92A, 92B having a different wavelength. This allows the cells to be exposed by different wavelengths of radiation to allow cells having different properties to be detected, for example through the use of alternative markers.
- 10 This also allows the processing system to use the imaging system 11 to determine from the colour of radiation exposing each cell 71, 72, which pipette 33A, 33B is adjacent the cell. This also allows cells 71, 72 having different predetermined properties to be detected, by arranging for each cell to respond to a respective wavelength of radiation, for example by the use of appropriate labels.
- 15 This aids in automating the system and provides for a method that allows a number of cell pairs to be rapidly fused as follows:
  1. Multiple cells in a source well are exposed to radiation from the LED 92A;
  - 20 2. Cells 71 having predetermined properties are detected by the processing system 10 and drawn into and stored in the pipette 33A, as shown in Figure 14.
  3. Multiple cells in a source well are exposed to radiation from the LED 92B;
  4. Cells 72 having predetermined properties are detected by the processing system 10 and drawn into and stored in the pipette 33B, in a similar fashion.
  - 25 5. Both pipettes 33A, 33B are inserted into a fusion well 44.
  6. A respective one of each cell type 71, 72, is expelled from each pipette 33A, 33B at the same time, such that hydrodynamic forces draw the cells 71, 72 together as shown in Figure 15.
  7. The processing system detects the positions of the cells using the imaging system 11
  - 30 such that when the cells are expelled from the respective pipette 33A, 33B the fluid flow is truncated.

- 35 -

8. A DEP electric field is applied to draw the cells together between the electrodes, as shown for example in Figure 12. At this point the cells are pushed together using an increased (amplitude) DEP field to aid membrane contact.
9. The field generator 13 applies a predetermined pulse sequence to the cells 71, 72 via the electrodes 90A, 90B.
10. The cells are again pushed together using an increased (amplitude) DEP field to aid in fusion.
11. The pipettes 33A, 33B move to a new position within well.
12. Steps 6 – 11 are repeated as many times as necessary, until a number of fusates 73 are provided as shown in Figure 16.
13. When all cell pairs have been expelled/fused on of the pipettes travels back through the well recovering the fusates.
14. Fusates are recovered to recovery wells either as single clones or groups.

15 A further development, is for an alternative form of actuator to be used. An example of this form of actuator is shown in Figures 17A, 17B.

As shown, in this example, the tube 54 is connected via a stopcock 62 and a reservoir 63 to a pump 64. An actuator 65 is positioned adjacent the flexible tube 54, to allow the tube to be clamped as shown in Figure 17B.

20 It will be appreciated from this that any form of actuator, such as a solenoid, may be used. However, in this example, the actuator is formed from a threaded screw drive 66, coupled a DC or stepper motor 67, which forms part of the drive system 32. In use, this allows the actuator to be moved in the direction of the arrow 69, an amount of  $\pm 5\text{mm}$ .

25 The actuator tip can have a piezo electric stack 68 coupled thereto, to allow fine control (displacement of  $\pm 20\mu\text{m}$ ) of the end of the actuator. Again, the piezo stack forms part of the drive system 32.

30 In use, the pipette is loaded with a suitable fluid medium by placing the aperture 58 into a container that has sufficient fluid to fill the system. The pump or other such means of

- 36 -

drawing fluid through the system is activated and fluid is drawn through the pipette nozzle 57. When the system is loaded and there are no air bubbles present in the tubing, the stopcock 62 is closed to prevent further fluid flow, and the pump 64 turned off.

- 5 Whilst the aperture 58 is still immersed in the fluid medium, the actuator 65 is adjusted such that the silicon tubing 54 is compressed to about half its diameter, as shown in Figure 2B. Thus, in use, with the port 41 positioned in fluid in a well causing the actuator 65 to move in the direction of the arrow 69 compresses or releases the tubing 54 which, in turn, either expels or draws in fluid through the port 41. This allows cells to be recovered from  
10 a well as described above with respect to the pipette of Figure 3.

- Accordingly, the system described above allows individual cells to be easily fused. As the cells are manipulated using the pipette as shown in Figure 3, this makes the cell manipulation far easier than in the prior art. This therefore helps increase the speed and  
15 ease with which fusion of individual cells can be performed. Furthermore, the electrodes need never touch the cells, thereby helping reduce or prevent cell damage prior to or during the fusion process.

- In addition to this, the apparatus as a whole is generally less complicated, thereby helping  
20 reduce the cost, as well as easing use of the apparatus to perform cell fusion. As a result, fusion using the system described above can generally be achieved more rapidly and cheaper than in the prior art.

- Persons skilled in the art will appreciate that numerous variations and modifications will  
25 become apparent. All such variations and modifications which become apparent to persons skilled in the art, should be considered to fall within the spirit and scope that the invention broadly appearing before described.

- Accordingly, while the above description has focused on cell fusion, it will be appreciated  
30 that the techniques may generally be applied to any cells, vectors, particles, molecules, liposomes, and other such vesicles.

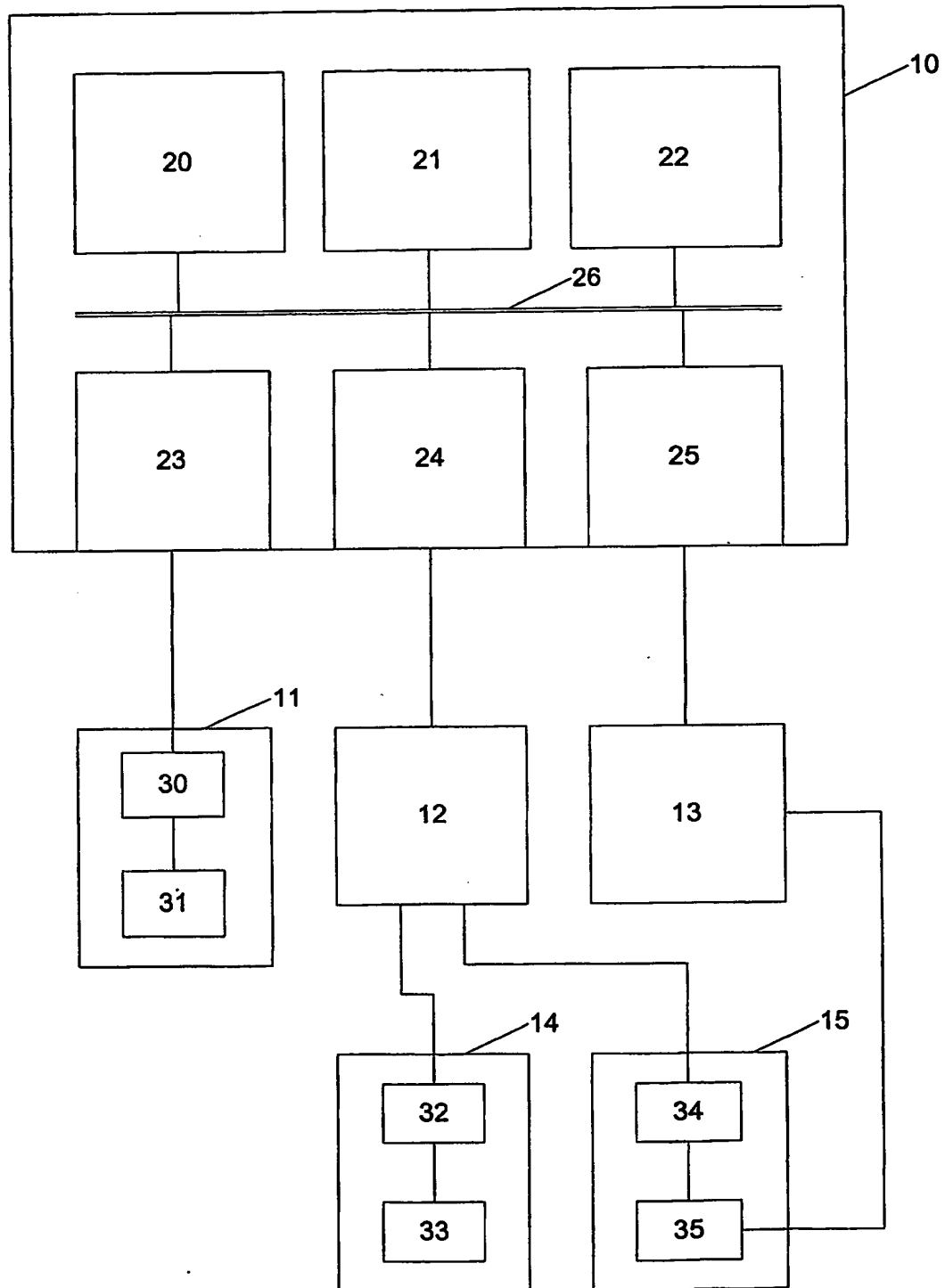
- 37 -

Dated this Fifth Day of September, 2002

**APOLLO LIFE SCIENCES PTY LIMITED**

5 By their Patent Attorneys

**DAVIES COLLISON CAVE**



**Fig. 1**



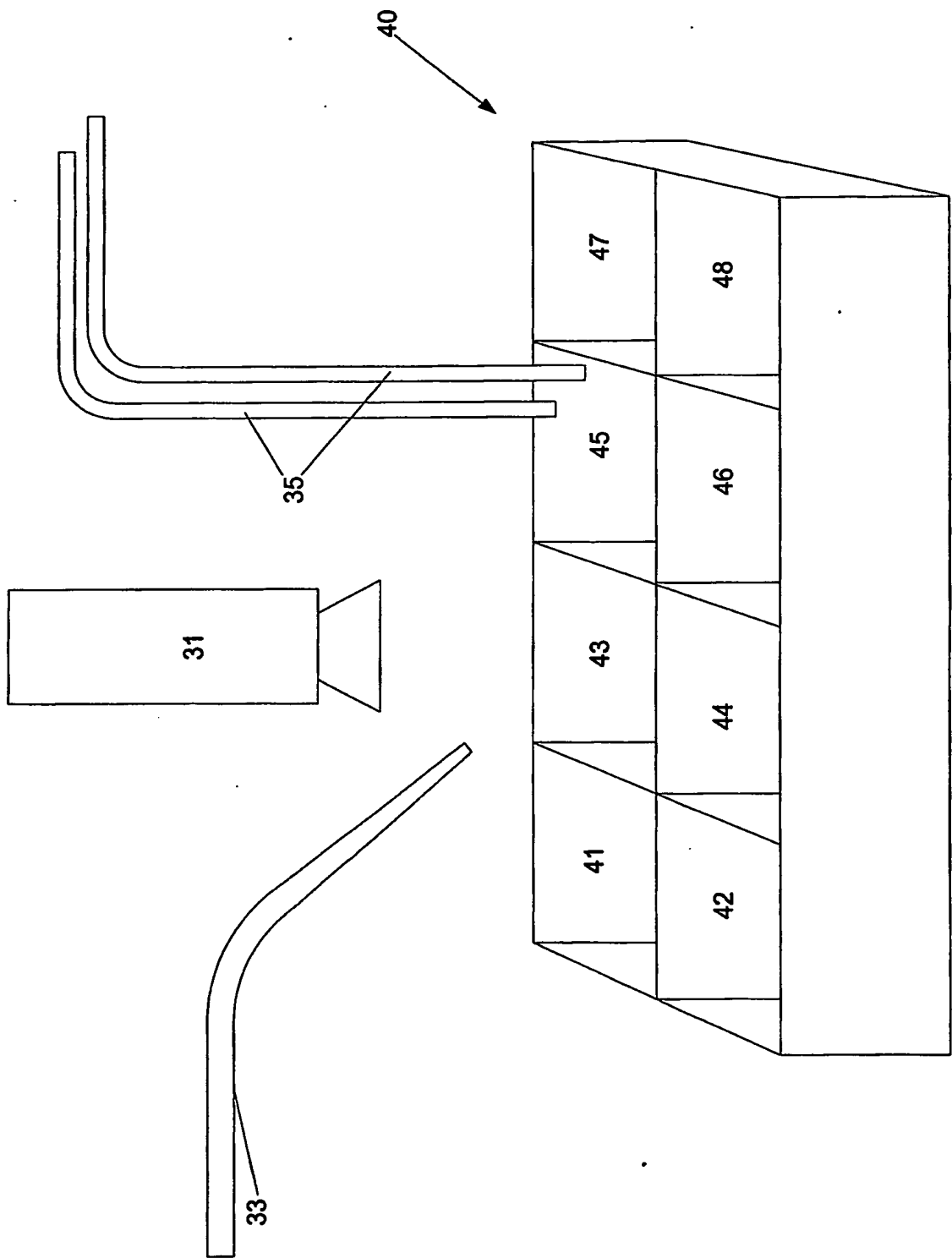
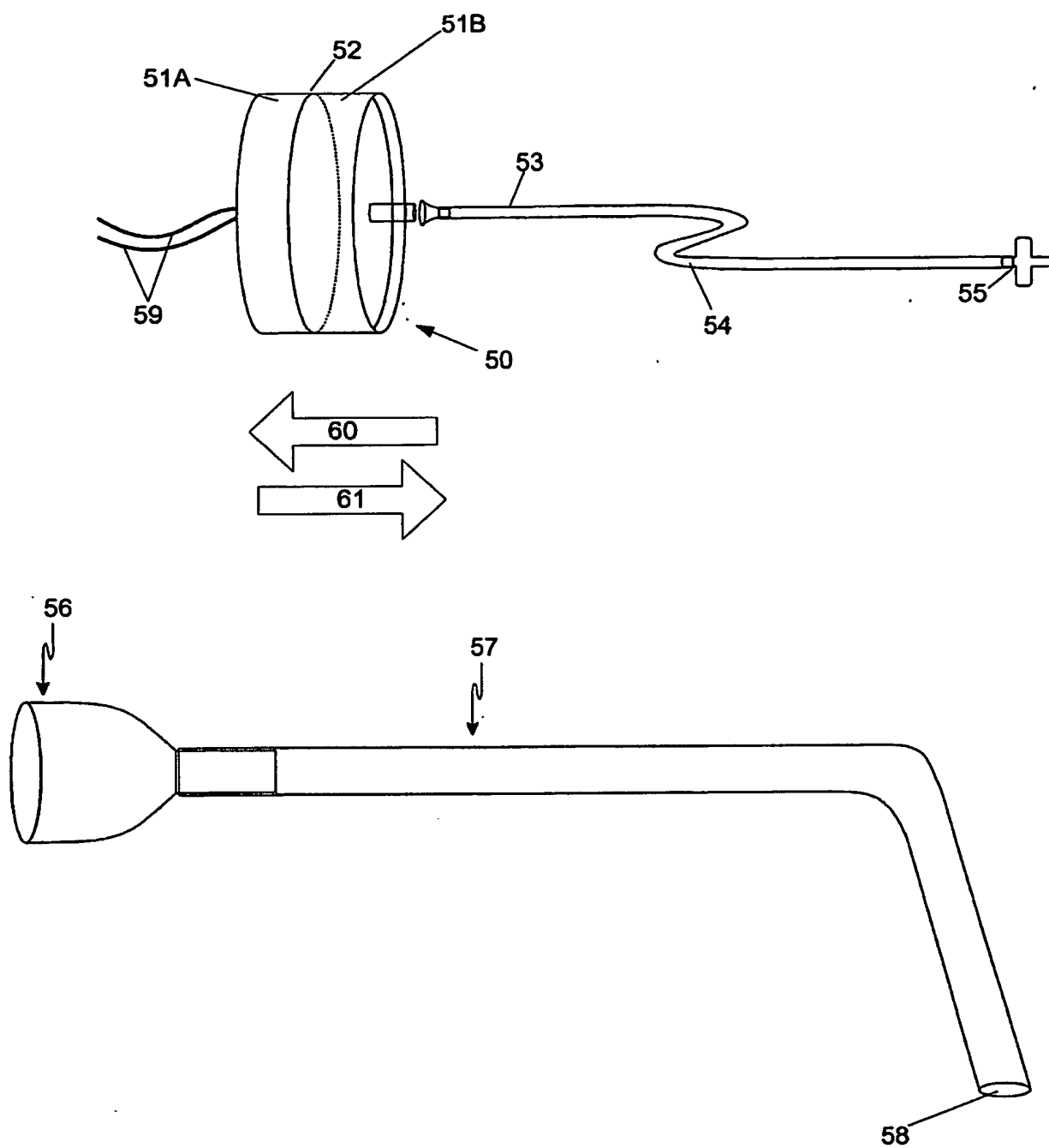
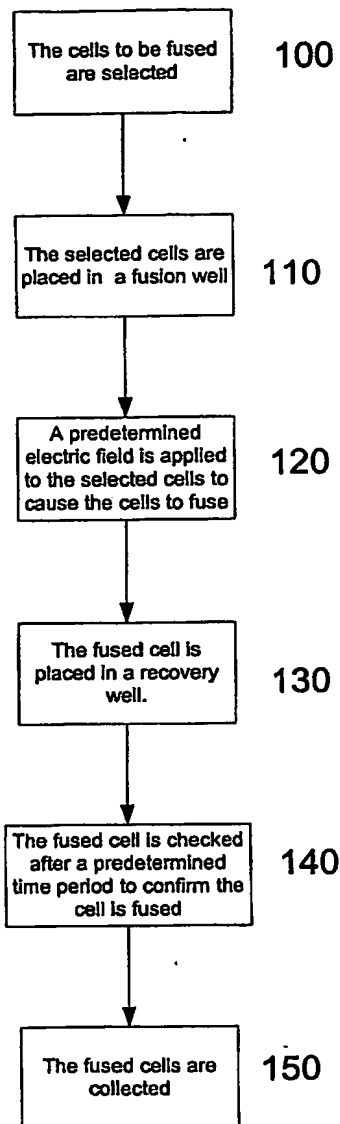


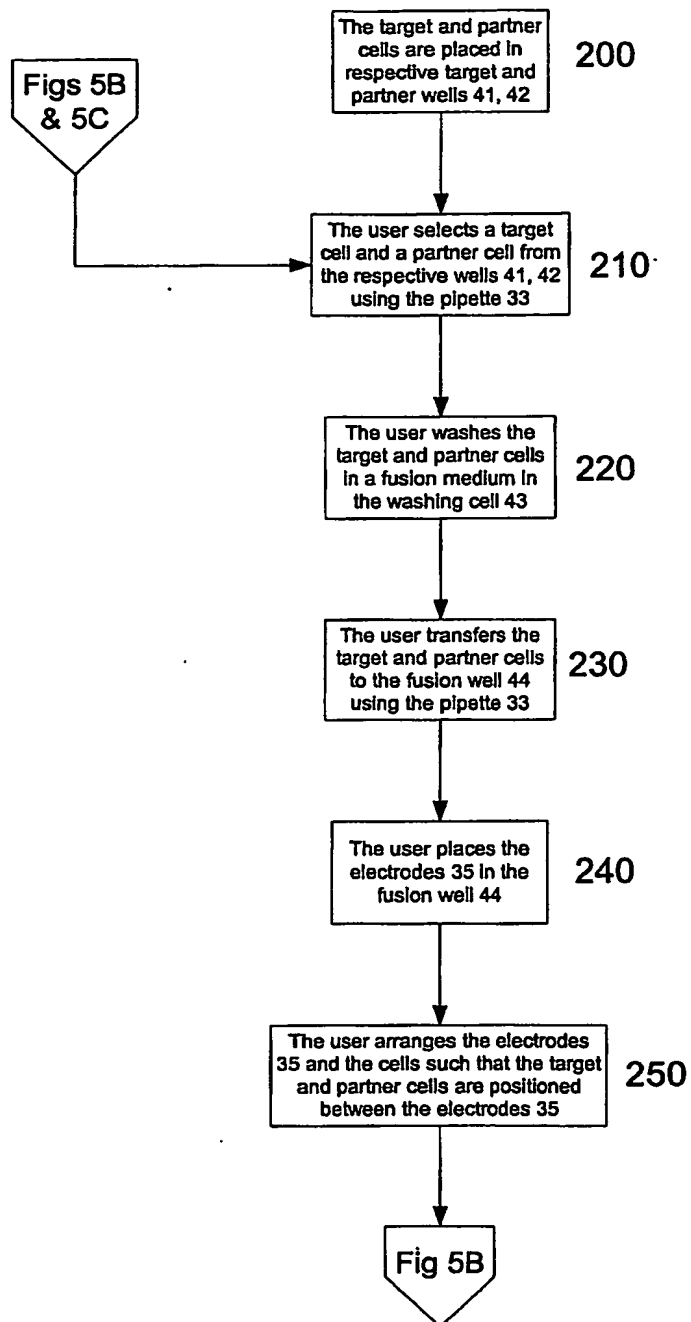
Fig. 2



**Fig. 3**



**Fig. 4**



**Fig. 5A**

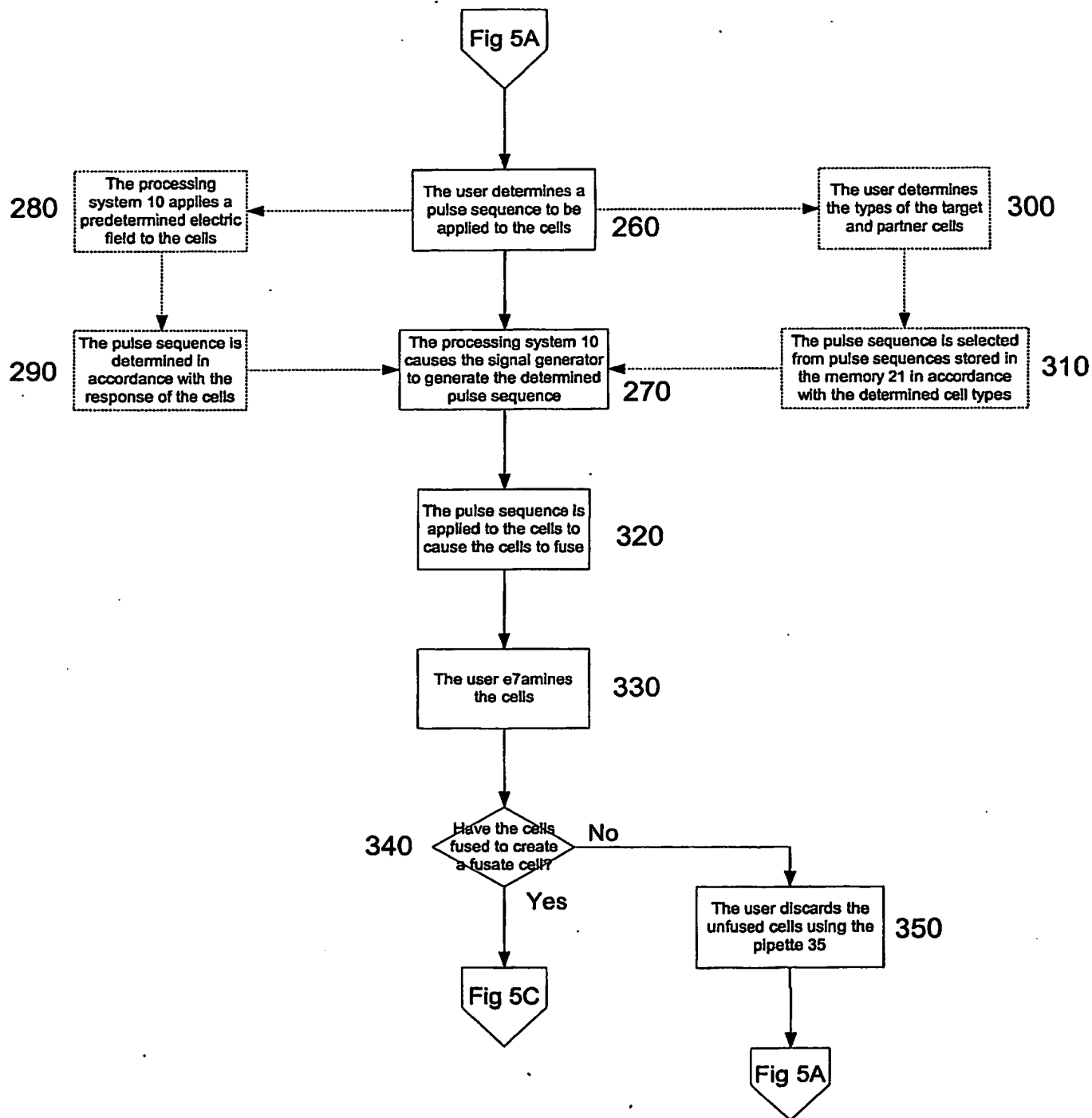
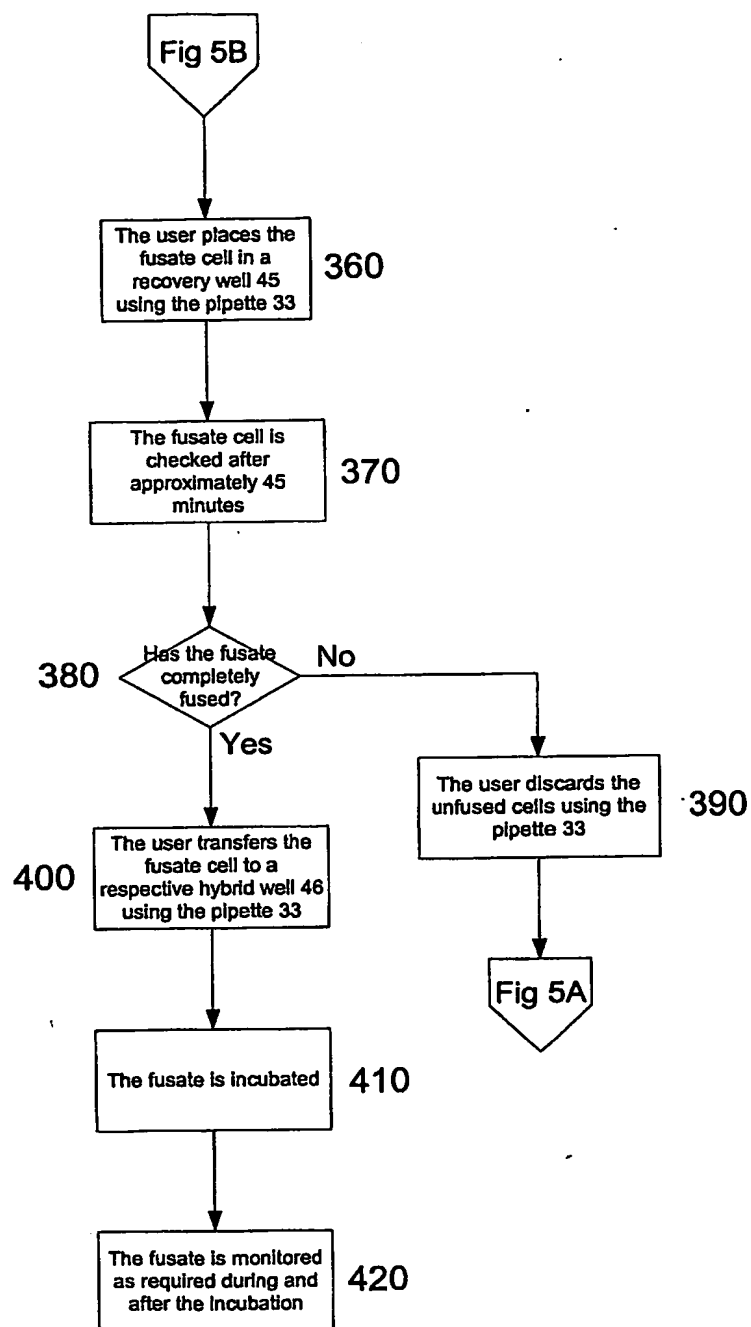


Fig. 5B



**Fig. 5C**

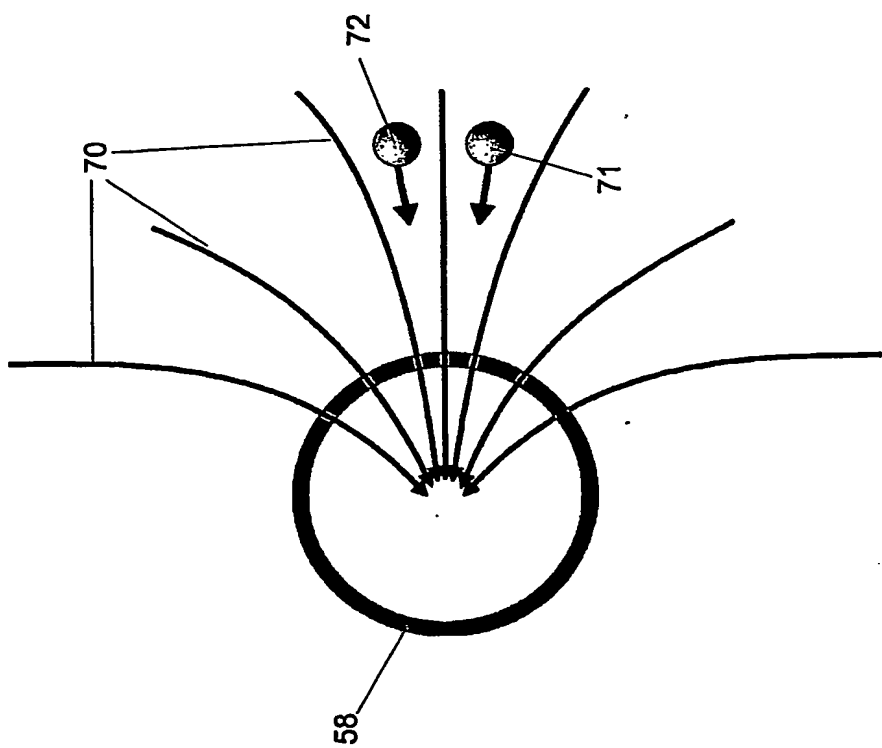


Fig. 6B

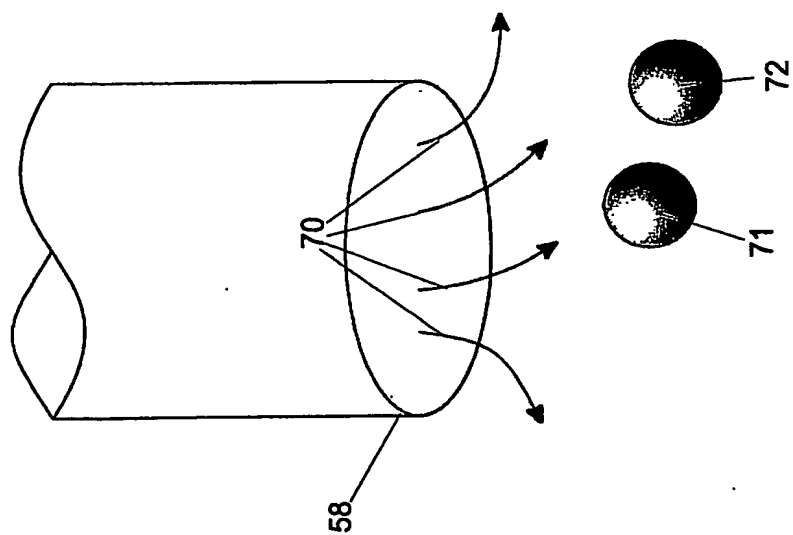


Fig. 6A

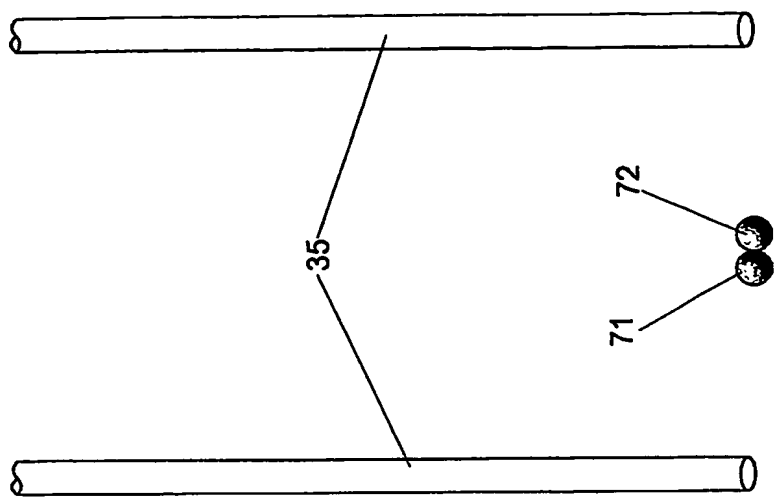


Fig. 6D

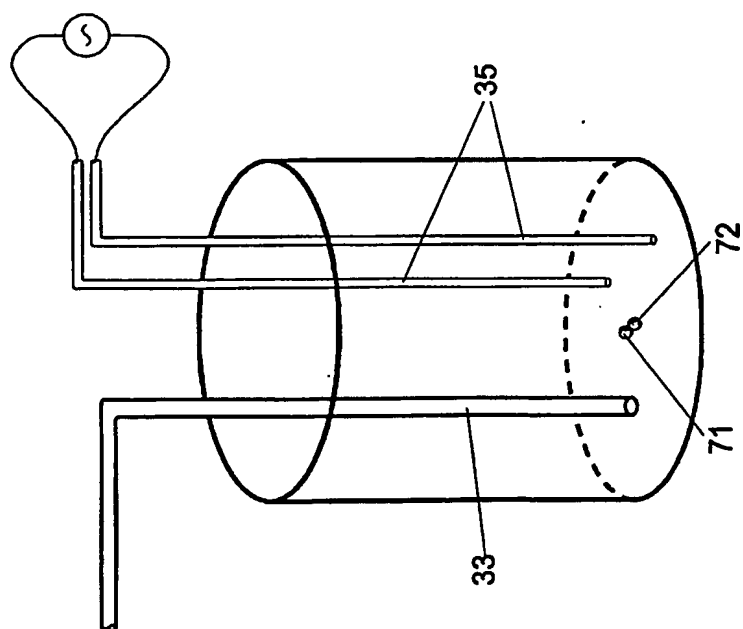
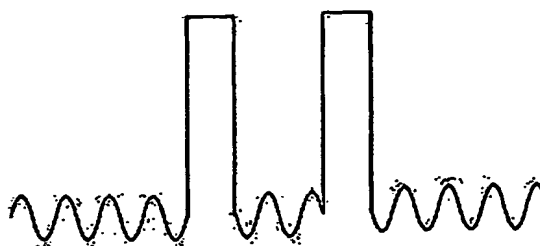


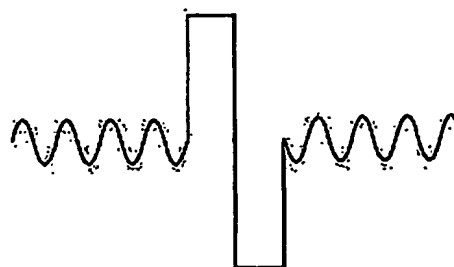
Fig. 6C



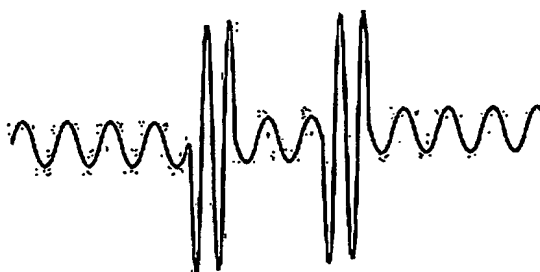
**Fig. 7A**



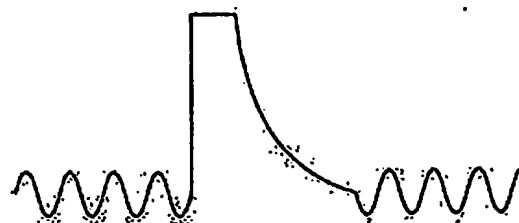
**Fig. 7B**

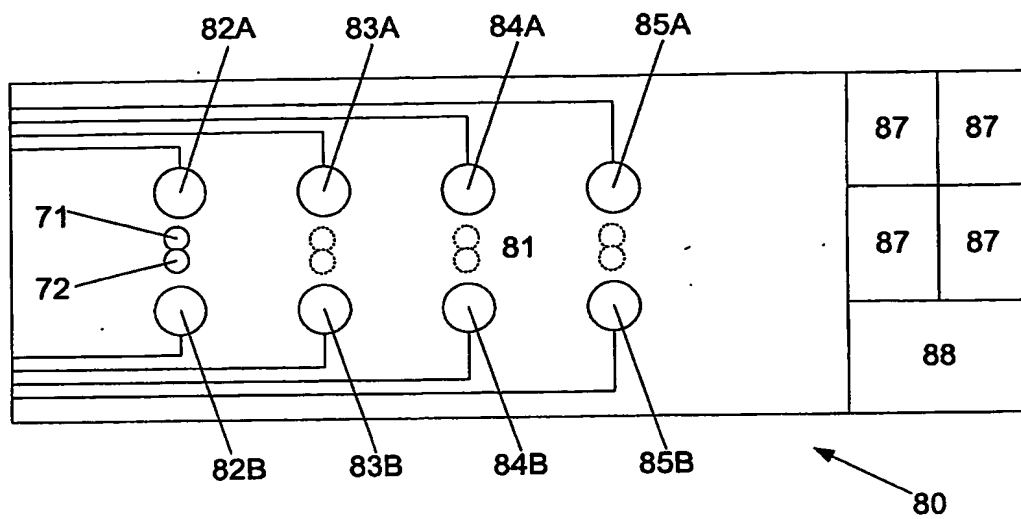


**Fig. 7C**

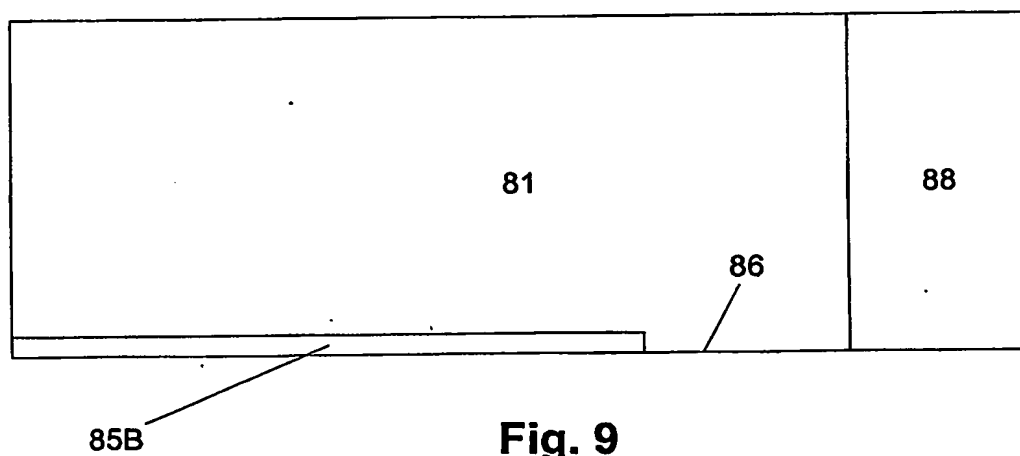


**Fig. 7D**

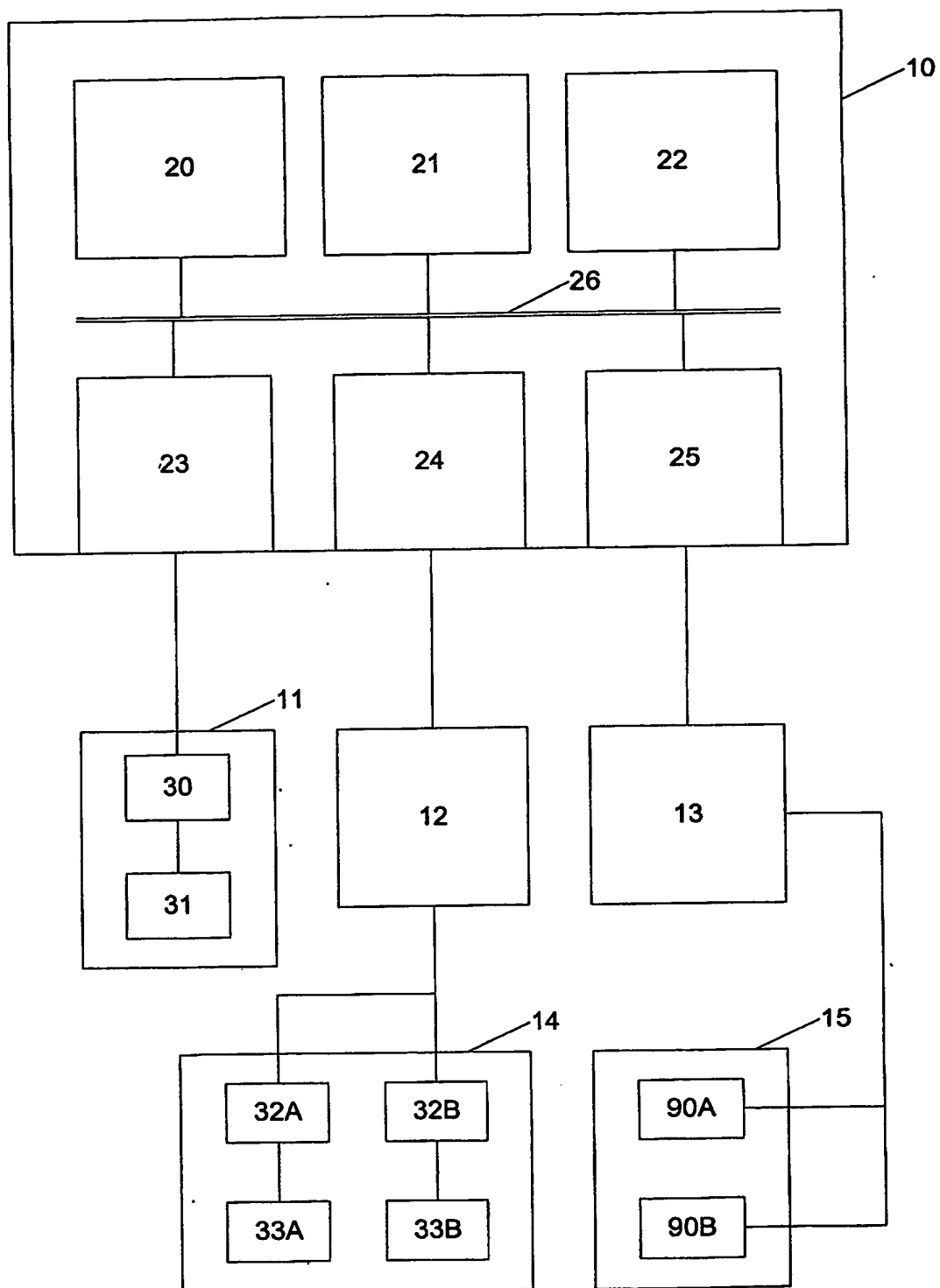




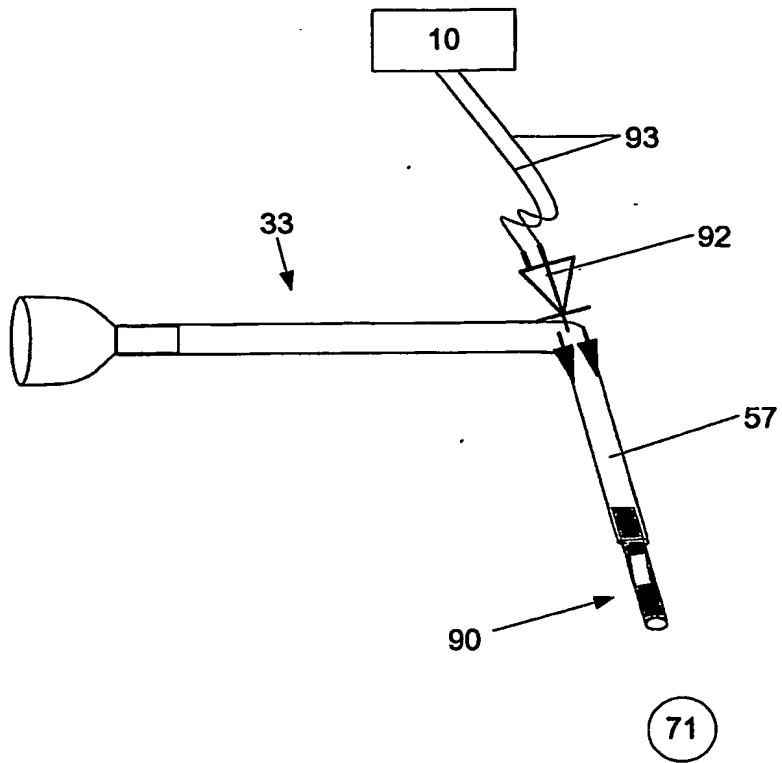
**Fig. 8**



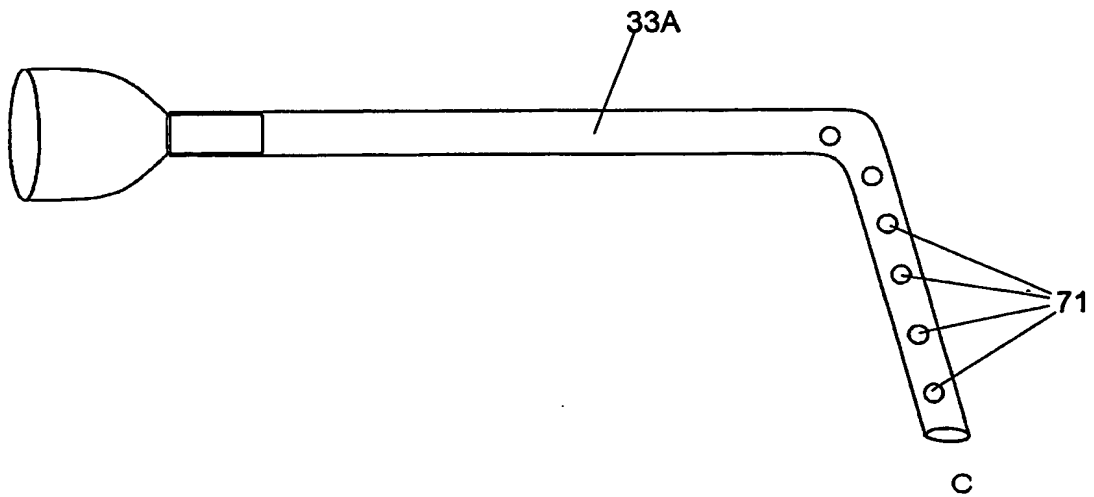
**Fig. 9**



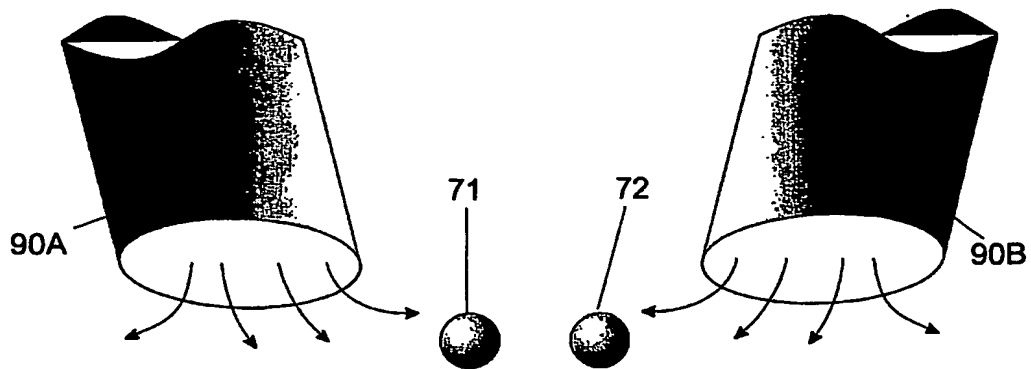
**Fig. 11**



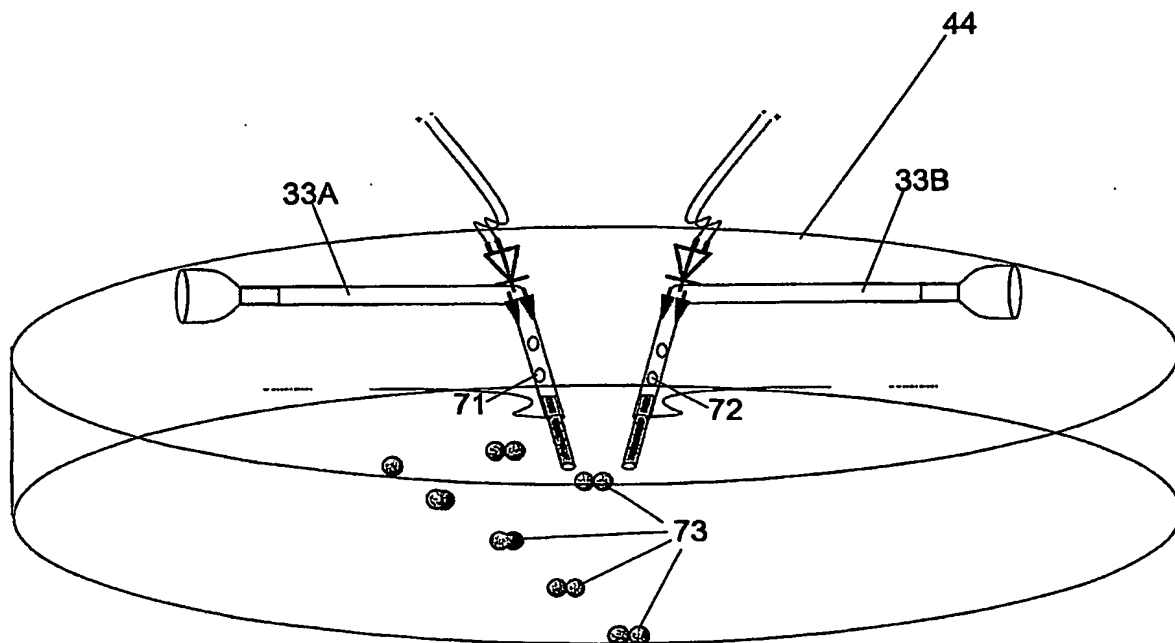
**Fig. 13**



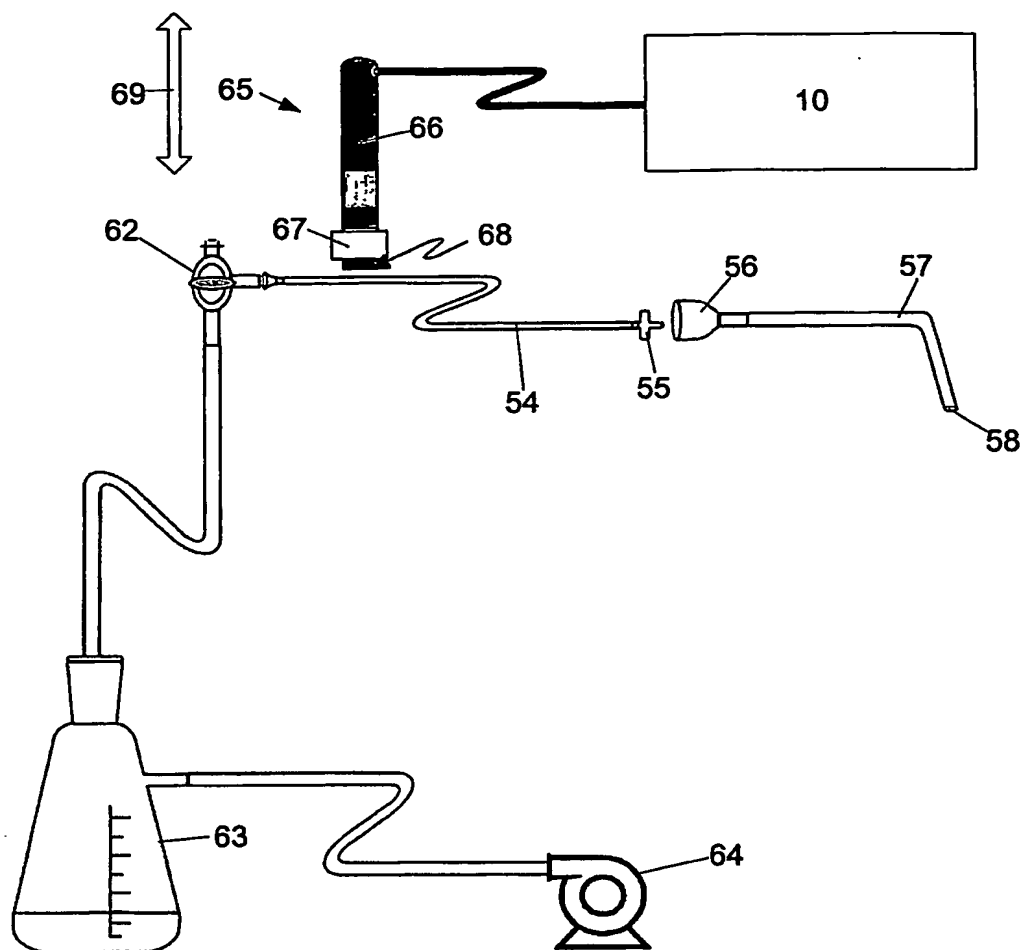
**Fig. 14**



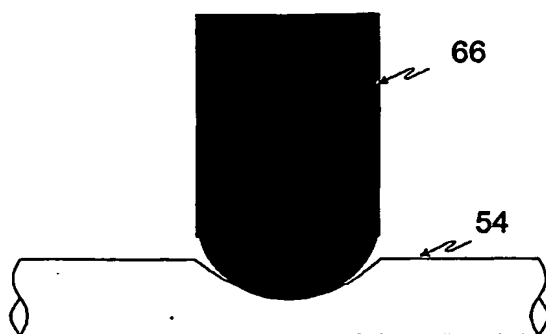
**Fig. 15**



**Fig. 16**



**Fig. 17A**



**Fig. 17B**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**